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Perch liver reaction to *Triaenophorus nodulosus* plerocercoids with an emphasis on piscidins 3, 4 and proliferative cell nuclear antigen (PCNA) expression

B.S. Dezfuli[a,*], L. Giari[a], M. Lorenzoni[b], M. Manera[c], E.J. Noga[d]

[a] *Department of Life Sciences and Biotechnology, University of Ferrara, St. Borsari 46, 44121 Ferrara, Italy*
[b] *Department of Cellular and Environmental Biology, University of Perugia, St. Elce di Sotto 5, 06123 Perugia, Italy*
[c] *Faculty of Biosciences, Agro-Alimentary and Environmental Technologies, University of Teramo, Italy*
[d] *Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 St. Hillsborough, Raleigh, NC 27606, USA*

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ABSTRACT

Histopathological lesions caused by plerocercoids of *Triaenophorus nodulosus* within the liver of perch, *Perca fluviatilis*, from Lake Trasimeno were studied. Livers harbored 1–3 parasite larvae and pathological alterations were more marked in those with 3 plerocercoids. In the liver, larvae were encysted, surrounded by a capsule of host tissue; two of 14 plerocercoids were necrotic. In infected livers, some hepatocytes showed degenerative changes, i.e. swelling and hydropic degeneration, notably those in close proximity to larvae. By comparison, hepatocytes in uninfected livers or in regions away from the point of infection appeared normal. The occurrence of macrophage aggregates (MAs) distributed among the mast cells (MCs) was observed around the encysted larvae. The cellular elements involved in the immune response within liver were assessed by immunohistochemical techniques and by the use of antibodies against the antimicrobial peptides piscidins 3 and 4, which revealed a sub-population of positive MCs. In infected livers, numerous MCs that were immunopositive to P4 and a few that were positive to P3 were found around *T. nodulosus* larvae. Histological sections of both uninfected and infected liver were immunostained with proliferative cell nuclear antigen (PCNA) antibody. Within the capsule and in close proximity to the parasite larvae, various cell types (i.e., MCs, fibroblasts and epithelioid cells) and a significantly higher number of PCNA-positive hepatocytes that were immunoreactive to PCNA were found compared to uninfected livers (ANOVA, $P < 0.05$). No parasites of any type were found in gill, spleen, kidney or gonad of *P. fluviatilis* and the intestine of 3 perch were infected with few specimens of *Acanthocephalus lucii*.

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1. Introduction

The fish tapeworm *Triaenophorus nodulosus* has a worldwide distribution that is closely correlated with the distribution of its definitive host, the pike, *Esox lucius*. This parasite is currently recorded in almost all waters of Europe where pike normally occurs. *T. nodulosus* inhabits the intestine of pike, uses copepods as first intermediate hosts, and a broad range of fish species (>70 spp.) as second intermediate hosts (e.g., European perch, *Perca fluviatilis*, see Kuperman, 1973; Kuchta et al., 2007). In European perch, plerocercoids are localized in the liver but are occasionally seen in other organs such as spleen, gonad, kidney and musculature (Kuperman, 1973; Brinker and Hamers, 2007). Controversial data exists on the effects of *T. nodulosus* plerocercoids on European perch growth and condition

* Corresponding author. Tel.: +39 0532 455701; fax: +39 0532 455715.
E-mail address: dzb@unife.it (B.S. Dezfuli).

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(Hoffmann et al., 1986; Brinker and Hamers, 2007). In European perch, the liver is the main site of infection; this organ is a nutrient-rich environment, an important reticulo-endothelial tissue, and metabolizes many drugs and other compounds (Hrckova et al., 2010). In mammals, such as mice, a fibrous capsule serves as a mechanical barrier to migration of liver-residing cestodes to other organs (Hrckova et al., 2010).

In European perch liver, *T. nodulosus* plerocercoids become encapsulated by a host tissue response (Brinker and Hamers, 2007), the walls of the capsule consisting of thick connective tissue and epithelioid cells (Hoffmann et al., 1986). In response to infection, a variety of cells become activated and cooperate in an effort to control and eliminate the invading pathogens (Makepeace et al., 2012). In fish, the innate defences responding to helminth infection commonly involve eosinophilic granular cells (Secombes and Chappell, 1996; Reite and Evensen, 2006; Buchmann, 2012; Dezfuli et al., 2012b, 2013) also named mast cells (MCs) (Ellis, 1985; Reite, 1997) and macrophage aggregates (MAs) or melanomacrophage centers (Agius and Roberts, 2003). A key factor of the piscine immune system is a group of antimicrobial peptides (AMPs) named piscidins, having potent, broad-spectrum antimicrobial activity against viruses, bacteria, fungi, water molds and parasites (Silphaduang and Noga, 2001; Park et al., 2011; Salger et al., 2011; Zahran et al., 2012).

Little is known about the immune response of fish to larval cestodes and no record exists on expression of proliferating cell nuclear antigen (PCNA) in liver of fish infected with a helminth. Alterations in the expression of PCNA have recently been applied to the fields of fish parasitology (Dezfuli et al., 2012a) and to fish health (Blas-Machado et al., 2000; Kong et al., 2008; Chikwati et al., 2013). Indeed, only few papers have been published on piscidins in fish-metazoan systems (see Dezfuli et al., 2010a, 2011a). Thus, the current study represents the first record of piscidins (P3, P4) and PCNA-positive cells within the liver of fish infected with a tapeworm. Emphasis will be placed on the role of MCs as an important component of the host's innate immune system.

2. Materials and methods

In July 2012, a total of 54 European perch, *P. fluviatilis* (19.74 ± 2.68 cm, mean total length $\pm$ standard deviation, SD; 107.94 ± 30.45 g, mean weight $\pm$ SD), were processed from Lake Trasimeno (Province of Perugia, Central Italy). The fish were caught by gill net that was deployed on one occasion by professional fishermen belonging to a local fishing consortium. Immediately upon landing, the fish were transferred alive to the consortium's facility where they were euthanized using an overdose of 125 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland). Thereafter, the spinal cord was severed and the fish measured and weighed. Upon post mortem, the fish were sexed before the digestive tract and other organs were removed in search of helminths. Pieces of infected liver (15 mm $\times$ 15 mm) were fixed in Bouin's fluid for 10 h and thereafter, were rinsed in several changes of 4 °C 70% ethanol before being stored in the same medium until they were processed for histology. The fixed tissues were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor. After blocking out, 5 μm thick sections were taken from each tissue block, stained with haematoxylin and eosin (H&E) and/or alcian blue 8 GX pH 2.5 combined with periodic acid Schiff's reagent (AB/PAS).

For tapeworm species and larval stage identification purposes, some live larvae were fixed in hot 10% formalin, examined *in vitro*, and identified on the basis of relevant literature (Kuchta et al., 2007).

Some histological sections were subjected to an indirect immunohistochemical method (peroxidase–anti-peroxidase immunocomplex) using anti-piscidin 3 (anti-HAGR) and anti-piscidin 4 (anti-5.3-02-3A) antibodies. The two primary antibodies against piscidins were produced by a commercial laboratory (Bethyl Laboratories, Montgomery, Texas, USA) using the company's standard procedures which are detailed in Dezfuli et al. (2010a) and Corrales et al. (2010). Briefly, sections (5 μm) were de-paraffinised in xylene, rehydrated through a graded alcohol series, then endogenous peroxidase activity and non-specific staining were blocked in 3% H_2O_2 for 10 min and then in normal goat serum (1:20, Elite Rabbit IgG Vectastain ABC Kit, Vector, Burlingame, USA) for 30 min. After incubation with the primary antibodies (anti-HAGR diluted 1:400 and anti-5.3-02-3A 1:8000) for 3 h at room temperature (RT), the sections were incubated for 30 min with a biotinylated goat anti-rabbit serum (Elite Rabbit IgG Vectastain ABC Kit, Vector), and then for 30 min with avidin-conjugated horseradish peroxidase (Elite Rabbit IgG Vectastain ABC Kit, Vector). The sections were then developed using DAB (3,30-diaminobenzidine 0.04% w/v in TBS 0.05 M, pH 7.4) and H_2O_2 (0.005%), rinsed and then counterstained with alcian blue and Harris's haematoxylin. Non-immune serum and diluent-only sections were used as negative controls. The positive control tissue was hybrid striped bass (*Morone saxatilis* $\times$ *M. chrysops*) intestine. The specificity of the reaction was confirmed by pre-absorption of each antiserum with the corresponding antigen.

Additional sections were subjected to the IHC method using a commercially available anti-PCNA antibody (PC10 sc-56 mouse monoclonal antibody, Santa Cruz Biotechnology, Inc.). After dewaxing in xylene and rehydrating through a graded alcohol series, the sections were treated for antigen retrieval in a citrate buffer (pH 8.0) for 20 min in a steam bath at 95 °C; thereafter, the slides were left for 10 min to cool to RT. Endogenous peroxidase activity and non-specific staining were blocked, respectively, in 3% H_2O_2 for 10 min and then in horse normal serum (1:20, Elite Mouse IgG Vectastain ABC Kit, Vector, Burlingame, USA) for 30 min. Sections were then incubated with the primary antibody (anti-PCNA diluted 1:500) for 2 h at RT. After washing with PBS, the slides were incubated for 30 min with biotinylated horse anti-mouse serum (Mouse IgG Vectastain ABC Kit, Vector) followed by avidin-conjugated horseradish peroxidase (Mouse IgG Vectastain ABC Kit, Vector). The enzyme activity was detected using DAB. Non-immune mouse serum and diluent-only sections were used as negative controls. The sections were then dehydrated, counterstained with alcian blue and Harris's haematoxylin.

Sections were examined and photographed using a Nikon Microscope ECLIPSE 80i.

For comparative purposes, liver sections from nine cestode-infected and eleven uninfected fish were screened for PCNA-positive cells via light microscopy using computerized image analyser software (Nis Elements AR 3.0). In each liver slide, i.e. in each fish, the ratio of PCNA-positive nuclei was determined by scoring 1000 hepatocytes in two randomly selected fields examined at ×400 magnification. In infected specimens, the nuclear count was made in the tissue immediately surrounding the cestode larva. The number of PCNA-positive nuclei was assessed for normality by means of the Kolmogorov-Smirnov and Shapiro–Wilk tests. A one-way ANOVA was performed to detect significant differences in the number of positive hepatocytes in the uninfected and infected livers. The statistical package Statistica 7 and a $P < 0.05$ level of significance were used throughout.

3. Results

3.1. Histopathology

No parasites of any type were found in gill, spleen, kidney or gonad of 54 *P. fluviatilis* but, the intestine of 3 hosts were infected with low number of *Acanthocephalus lucii*. However, nine of infected perch (16.6%) harbored *T. nodulosus* plerocercoids in liver (Fig. 1a). Infection intensity ranged from 1 to 3 plerocercoids per liver (mean infection intensity ± SD, 1.55 ± 0.88), with most larvae encysted under the peritoneal visceral serosa; a few larvae penetrated the parenchyma and damaged the liver tissue. Histological sections of infected livers revealed that two encysted plerocercoids out of 14 were necrotic; in two infected livers, the necrotic plerocercoid was close to a live larva.

Most plerocercoids were encapsulated on the surface of the liver, enclosed by a granulomatous response involving the peritoneal visceral serosa (Fig. 1a). The wall of the capsule was composed of two layers (Fig. 1b): the innermost, which was adjacent to the plerocercoid consisted of host connective tissue, mainly collagenous fibres, whilst the second, outer layer consisted of mast cells (MCs), fibroblasts and epithelioid cells (Fig. 1c and d). Macrophage aggregates (MAs) were observed in the outer part of the second layer (Fig. 1b and d). Within infected livers, several hepatocytes showed degenerative changes, notably those in close proximity to a plerocercoid. Histologically, these were recognizable as swollen and in a hydropic degenerative state (i.e. cellular oedema, Fig. 1e). The hepatocytes in uninfected livers and in the tissues of infected perch distant from the site of cestode infection, appeared normal (Fig. 1f).

3.2. Immunohistochemistry

Within the capsule enveloping a cestode larva, numerous cells (MCs, fibroblasts and epithelioid cells) that were PCNA positive (i.e. proliferating) were documented (Fig. 2a and b). The number of PCNA-positive hepatocytes in infected liver, close to the site of *T. nodulosus* infection, were significantly higher than the number observed in uninfected liver (ANOVA, $P < 0.05$).

Serial sections treated with anti-piscidin 3 (P3) and anti-piscidin 4 (P4) antibodies showed that sub-populations of MCs were positive within the capsule of infected liver. Within the outer layer of the capsule, very few MCs were positive for P3 (Fig. 2c). P4-positive MCs were more abundant than P3-positive cells within the outer layer of the capsule in infected liver (Fig. 2d and e). Piscidin-positive MCs were also found adjacent to, and in some instances, within, hepatic blood vessels (Fig. 2f).

4. Discussion

P. fluviatilis is widely distributed throughout the Palaearctic region and serves as a host to numerous endoparasitic helminths; its parasite fauna is relatively well known (Kuchta et al., 2007). As a common fishery species, it is of great economic importance in many lake systems throughout Europe (Kuchta et al., 2007).

There is no agreement as to the effect that plerocercoids of *T. nodulosus* have on European perch condition and growth. Hoffmann et al. (1986) suggested that there is no negative influence, although Dieterich and Eckmann (2000) indicated that the tapeworm could cause exceptional pathological alterations including total necrosis of the liver. In our study, most hepatocytes close to the cestode larvae were swollen and showed hydropic degeneration. However, we cannot from this finding alone ascertain the effect of the parasite on liver function, general physiological condition, or probability of survival.

For those fish species that act as either a paratenic or second intermediate host for several helminth taxa, the larvae can be found encysted in a range of different body organs (Ogawa et al., 2004; Santoro et al., 2013), and the magnitude of the subsequent host reaction can vary markedly. Typically, the response involves leucocytes, fibroblasts and collagenous connective tissue (Sharp et al., 1989; Dezfuli et al., 2007; Santoro et al., 2013) which surround the invading parasite with, according to some other authors, little, if any, host immune response (Stein and Lumsden, 1971; Galaktionov et al., 1997). The subsequent structure and formation of a capsule around a range of helminths infecting the viscera of different fish has been documented by several authors (Dezfuli et al., 2011b; Shamsi et al., 2011; Ahammed Shareef and Abidi, 2012; Santoro et al., 2013). One of the functions of the host connectival capsule is to sequester the parasite (Hrckova et al., 2010). A widely-held belief is that the main function of host immune system is to protect the organism against infection in order to minimize the fitness costs of being infected (Rohlenová et al., 2011).

The intense response documented in our investigation involved mast cells (MCs), fibroblasts and epithelioid cells positive for PCNA, MCs positive to piscidins 3 and 4, and macrophage aggregates (MAs). Most authors ascribe a role to MAs in the destruction, detoxification and recycling of endogenous and exogenous materials (Agius and Roberts, 2003; Mela et al., 2007; Passantino et al., 2013). It appears that MAs are involved with late stages of a chronic inflammatory response to severe tissue damage (Agius and

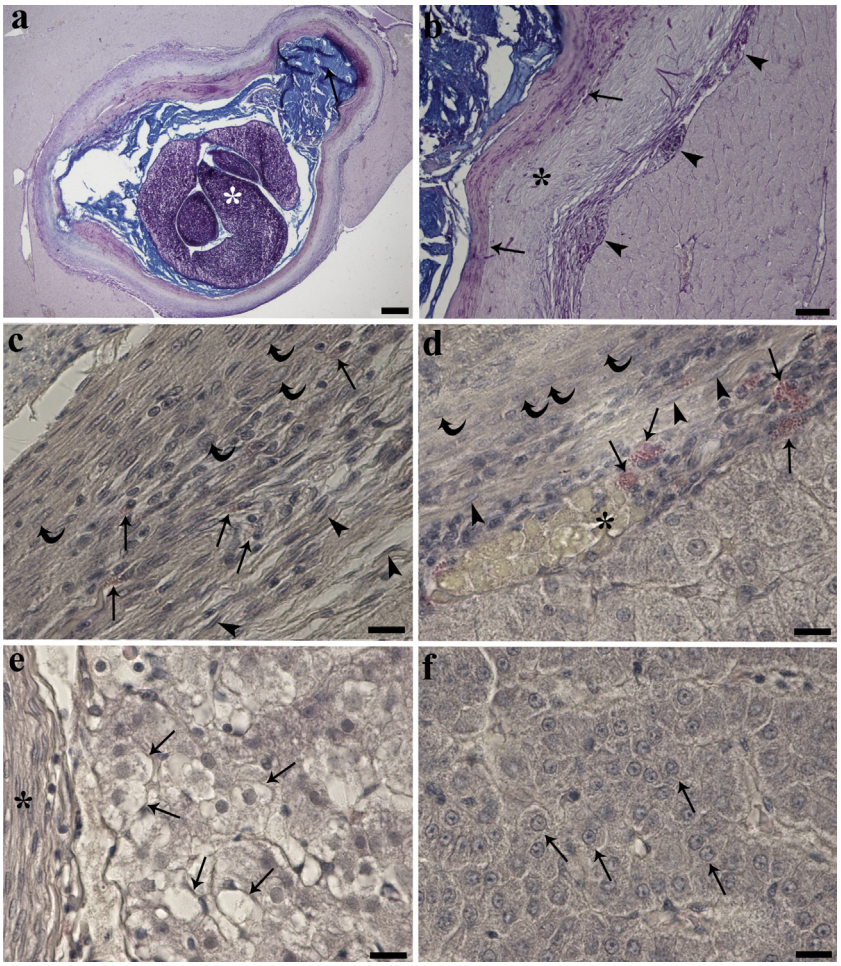

Fig. 1. *Triaenophorus nodulosus*-infected liver of European perch, *Perca fluviatilis*. (a) Sagittal section through the middle region of liver with an encysted plerocercoid (white asterisk), AB/PAS, bar = 200 μm. (b) Micrograph shows the capsule wall. The inner layer is highlighted by arrows and three macrophage aggregates (arrow heads) are evident in the outer part of the second layer (asterisk), AB/PAS, bar = 50 μm. (c) High magnification of the capsule wall in which mast cells (arrows), epithelioid cells (curved arrows) and fibroblasts (arrow heads) are visible, H&E, bar = 10 μm. (d) Micrograph of the outer layer of the capsule, where epithelioid cells (curved arrows), fibroblasts (thick arrows) and mast cells (arrows) in the vicinity of macrophage aggregates (arrow heads) can be seen, H&E, bar = 10 μm. (e) Infected liver, close to the capsule (asterisk) which surrounds a parasite. Here, the hepatocytes (arrows) appear swollen, displaying hydropic degeneration, H&E, bar = 10 μm. (f) Hepatocytes (arrows) from an uninfected liver, H&E, bar = 10 μm.

Roberts, 2003; Koppang et al., 2005; Dezfuli et al., 2007) and have roles in host innate and adaptive immunity (Wolke, 1992; Bols et al., 2001). Our investigations on the liver of flounder, *Platichthys flesus*, infected with nematode larvae (see Dezfuli et al., 2007), together with our observations from the current study, lend support to the view of Vogelbein et al. (1987) that MAs may be linked to parasite infections and, in all likelihood, represent an inflammatory response that is different from the typical granulomatous reaction. According to Vogelbein et al. (1987), in *Rivulus marmoratus*-infected with a coccidian (*Calyptospora funduli*), parasite-induced necrotic foci could form true granulomata in response to oocysts, or MAs in response to macrogamont degeneration.

Changes in expression of proliferating cell nuclear antigen (PCNA), a 36 kd protein involved in protein synthesis, can provide an early indication of changes in cell proliferation (Mathews et al., 1984) and can be detected via immunohistochemical staining (Ortego et al., 1994). PCNA immuno-positivity is confined largely to cell nuclei. Most observations of alterated PCNA expression in fish have studied general health condition or exposure to toxicants (Blas-Machado et al., 2000; Kong et al., 2008; Chikwati et al., 2013). Nevertheless, some information exists on the expression of PCNA in fish infected with the intestinal protozoan (Buret et al., 1990; Hemmer et al., 1998), but the relationship between PCNA and helminth infection was only recently studied. In brown trout (*Salmo trutta*) naturally infected with an intestinal helminth (acanthocephalan), we documented an increase in PCNA-positive epithelial cells, MCs and fibroblasts close to the site of parasite attachment (Dezfuli et al., 2012a). Our present immunohistochemical results demonstrated a significantly higher number of PCNA-positive hepatocytes in the liver of perch infected with *T. nodulosus* plerocercoids than in uninfected counterparts. This may explain the increase

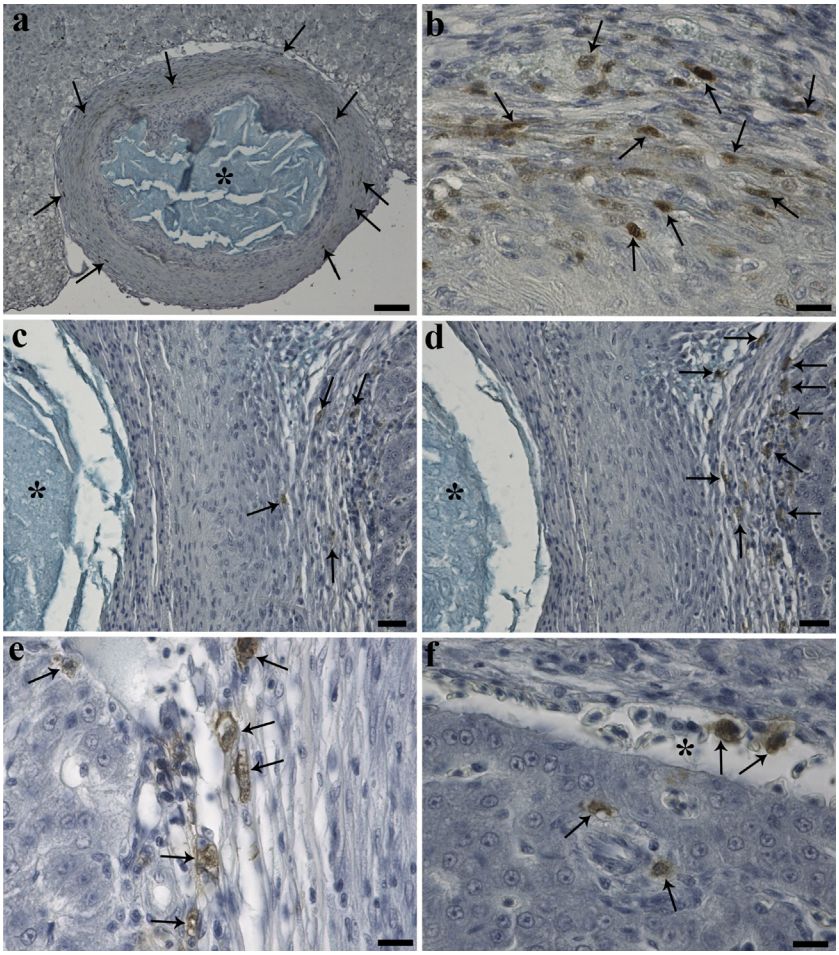

Fig. 2. *Triaenophorus nodulosus*-infected livers of *Perca fluviatilis*. (a) Sagittal section through a liver with an encysted plerocercoid (asterisk). Numerous cellular elements (arrows) within the capsule are positive to the PCNA antibody, bar = 50 μm. (b) High magnification of the PCNA-positive elements within the capsule, bar = 10 μm. (c) Piscidin 3-positive mast cells (arrows) close to the capsule which envelops a *T. nodulosus* plerocercoid (asterisk), bar = 20 μm. (d) From the corresponding serial section presented in c, numerous piscidin 4-immunoreactive mast cells (arrows) are visible. The asterisk highlights the position of the plerocercoid, bar = 20 μm. (e) High magnification of piscidin 4-positive mast cells (arrows) within an infected liver, bar = 10 μm. (f) Mast cells (arrows) that are positive to the piscidin 4 antibody inside the blood vessel lumen (asterisk) and within the parenchyma of an infected liver, bar = 10 μm.

in hepatocyte proliferation within infected liver which attempt to replace damaged cells around the plerocercoid. Interestingly, within the capsule enveloping the *T. nodulosus* larva, MCs, fibroblasts and epithelioid cells appeared to be immunopositive to the PCNA antibody.

Mast cells in fish are motile and differentiate in the haematopoietic organs, reaching their target tissues via the circulatory system as immature cells (Flaño et al., 1996). In infected European perch liver, numerous MCs were seen within the capsule, both on the outside and within capillaries. The close association of MCs with capillary endothelial cells suggests that they may move across the endothelium (Murray et al., 2007; Dezfuli et al., 2008, 2010b). The parasites may induce recruitment of MCs to the site(s) of infection (Alvarez-Pellitero, 2008) and stimulate their proliferation in situ. Many types of tissue injury, including pathogens, can activate MCs (Marshall and Jawdat, 2004).

In the current study, a high number of PCNA-positive cells were seen in the capsule surrounding the plerocercoid; most of these were MCs and fibroblasts. The co-occurrence of fibroblasts and MCs has been described from a range of fish species including rainbow trout (see Flaño et al., 1996), coho salmon, *Oncorhynchus kisutch* (see Kent et al., 1993), minnows, *Phoxinus phoxinus* (see Dezfuli et al., 2009) and brown trout (see Dezfuli et al., 2012a). MCs have the potential to directly influence fibroblasts and/or indirectly influence other cells, leading to a profibrotic response (Puxeddu et al., 2003), fibrotic process and tissue remodelling (Rocha and Chiarini-Garcia, 2007). The recruitment and proliferation of MCs around encysted plerocercoids may also serve to repair and remodel damaged hepatic tissue. The recruitment of MCs within sheep liver infected with *Fasciola hepatica* was reported by Ferreras et al. (2000) and Vukman et al. (2013), the same finding we observed. Our results are the first documenting PCNA-positive cells in liver of fish infected with a helminth.

Epithelioid cells are considered differentiated macrophages that form with persistent inflammatory stimulation and somewhat resemble epithelial cells. In fish, epithelioid cells with epithelial cell characteristics,

such as the presence of desmosomes, tonofilaments and cytokeratin positivity, have been reported and the involvement of other cells type, e.g. mesothelial cells, has been proposed in piscine chronic inflammatory response (Noga et al., 1989). In mammals, as reported by Papadimitriou and Spector (1971), young epithelioid cells can divide with an apparently similar proliferative capacity to macrophages. Furthermore, completely mature epithelioid cells are reported to divide in pulmonary lesions and not to divide in dermal lesions induced by Bacillus Calmette-Guérin (Ando et al., 1972; Shima et al., 1972). Such proliferative capacity has been previously tested with [^{3}H] thymidine (tritiated thymidine) labeling, the "gold standard" for cell kinetic studies and not subject to the possible bias known for PCNA, where positivity is sometimes described in absence of true cell division because of antigen labeling also occurs in G_1 and G_2 phases, not only in S phase (Hall and Levison, 1990). Thus, our current finding of the possible proliferative capacity of epithelioid cells agrees with those in mammals and deserves further study to fully elucidate its nature and function.

Antimicrobial peptides (AMPs) are natural antibiotics bestowed upon all forms of life (Ruangsri et al., 2012). One component of the innate immune system is AMPs which have a crucial role as a first-line host defence against pathogens (Silphaduang and Noga, 2001; Gordon et al., 2005). A group of AMPs in fish are the piscidins and they were isolated for the first time from commercially cultured, hybrid striped bass (Silphaduang and Noga, 2001). Piscidins have potent, broad-spectrum antimicrobial activity against several pathogens and parasites (Silphaduang and Noga, 2001; Park et al., 2011; Salger et al., 2011; Zahran et al., 2012). Very few papers have been published on piscidins in fish-metazoan systems (see Dezfuli et al., 2010a, 2011a). The current study represents the first record of piscidins (P3, P4) within cestode-infected liver of European perch, where the number of MCs that were immunopositive to P4 was higher than the number that were P3-positive. It is hoped that the histopathological and immunohistochemical results presented here will provide a basis for the future elucidation on piscine antihelminthic responses alongside observations on the immune cells of the liver and their role in the inflammatory response.

Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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